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Spectrophotometric Determination of Nickel (II) by Complexing with EDTA as Employed for Tracking its Loss in Immobilized Metal-ion Affinity Chromatography

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ABSTRACT:
A rapid spectrophotometric assay was developed to determine the Ni^{2+} capacity in Ni^{2+}-charged IMAC columns and measure the leached Ni^{2+} amount during IMAC. It was found that both Ni^{2+} and Ni-EDTA have unique UV-visible absorbance spectra from 350nm to 1000nm. Ni-EDTA has absorbance peaks at around 384nm, which is about three times stronger than free Ni^{2+} ion and is not interfered by excess amount of EDTA. By complexing Ni^{2+} with excess EDTA, it is possible to routinely measure the Ni^{2+} content in IMAC columns and measure the leached Ni^{2+} amount by comparing the Ni^{2+} contents before and after chromatography. The assay can be performed in either microplate format for A_{384} quantification or chromatography format for peak area comparison.

Key words: Ni^{2+}, Ni-EDTA, spectrophotometry, IMAC

INTRODUCTION

Immobilized metal-ion affinity chromatography (IMAC) is a widely used technique for purifying polyhistidine-tagged recombinant proteins. IMAC utilizes free co-ordination sites of immobilized transition metal ions to bind side chains of certain residues in proteins or peptides [1], as schematically illustrated in Figure 1.

When performing IMAC, it is necessary to determine the capacity of immobilized metal ligand in order to estimate the column efficiency. In addition, as chromatography proceeds, immobilized metal ion may leach from the column, which remains to be a major issue needing to be addressed. There are many factors contributing to metal ligand leaching, such as the chelating agents immobilized on IMAC resins, feed stream characteristics, and other chelating agents in buffers. Metal leaching could result in reduced ligand capacity, protein loss, and metal contamination of protein molecules, which is undesirable in purification. However, on the other hand, it allows regulation of metal ligand density, which could help to increase the binding selectivity for polyhistidine-tagged proteins. Several strategies have been reported to reduce the metal ion density, one of which is limited stripping of metal ions with low concentration of EDTA [2, 3].

While it is necessary to measure the metal ligand capacity in IMAC columns and monitor the ligand loss during chromatography, there are not many reports addressing this issue [4]. Among the transition metal ions, Ni^{2+} is widely used as IMAC binding ligand. In this study, a spectrophotometric method was developed for rapid measurement of Ni^{2+} capacity in Ni^{2+}-charged IMAC columns.

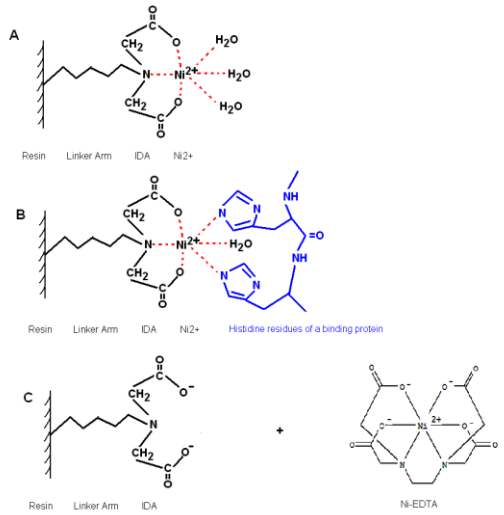

Figure 1. Schematic illustration of a Ni^{2+}-charged IMAC resin (A); Polyhistidine protein binds to Ni^{2+} through imidazole rings (B), and Ni^{2+} stripped by EDTA from IMAC resin (C). IDA: iminodiacetic acid

MATERIALS AND METHODS

The wavelength scanning experiments were carried out using a Cary 50 Bio UV-visible spectrophotometer (Varian, Inc., Palo Alto, CA). The following samples were scanned over the wavelength

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range from 200nm to 1000nm: 50mM $NiSO_4$, 50mM Mg-EDTA, 2x phosphate buffered saline (PBS) (without Ca^{2+}/Mg^{2+}), 2x PBS (without Ca^{2+}/Mg^{2+}) with 250mM imidazole, pH7.4.

The titration experiments were carried out as following: 5mM of Ni^{2+} was titrated with different concentrations of EDTA, namely 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50mM, and 5mM EDTA was titrated with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 30, 40, 50mM of Ni^{2+}. After each titration, the sample was subjected to wavelength scanning.

To develop the nickel assay, a flat bottom 96 well cell culture plate (Corning Inc., Corning, NY) was used. To allow formation of blue-colored Ni-EDTA, 100µl of sample was mixed gently with 25µl of 500mM EDTA for ~2 minutes, and the absorbance was measured at 384nm using a Spectra MAX 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Standards with Ni^{2+} concentration ranging from 0.1 to 50mM were used to build the standard curve, which gave good linearity. The detection limit was found to be ~400µM, and the quantitation limit at ~3mM. Samples were made as following: A HiTrap chelating Sepharose HP 5ml column (Amersham Biosciences, Piscataway, NJ) was sanitized and flushed extensively with de-ionized water, which was then charged with 2CV, or 10ml, of 50mM $NiSO_4$ solution, and washed with 2CV of de-ionized water and 5 – 10ml of PBS (without Ca^{2+}/Mg^{2+}). All the flowthrough and wash fractions were carefully collected and their volumes were measured. The column was either stripped directly with 2CV of EDTA or used to run chromatography with the following feed streams: PBS (without Ca^{2+}/Mg^{2+}), pH7.4, PBS (without Ca^{2+}/Mg^{2+}), pH7.4, in the presence of 2mM EDTA/5mM $MgSO_4$, which mimics the EDTA/Mg^{2+} treatment condition [3]; *E. coli* microfluidized whole cell extract. After chromatography, the column was stripped with 2CV of 500mM EDTA. All the fractions came out from the column were collected and analyzed.

RESULTS AND DISCUSSIONS

The wavelength scanning experiments showed that Ni^{2+} and Ni-EDTA have unique absorbance from 340nm to 1,000nm, compared to EDTA and other samples scanned, as shown in Figure 2. Ni^{2+} has an absorbance peak at 395nm, while Ni-EDTA has a much stronger absorbance peak at 384nm. By complexing Ni^{2+} with EDTA, it is possible to monitor the absorbance at 384nm for measuring the Ni^{2+} concentration. In fact, Ni^{2+} is usually stripped from the column by high concentration of EDTA, which results in formation of Ni-EDTA complex. Hence it is practical to convert Ni^{2+} into Ni-EDTA for the assay. Since EDTA itself does not have absorbance around 384nm, it was assumed that excess amount of EDTA, which occurs in column stripping, does not interfere with the absorbance of Ni-EDTA.

To demonstrate this, a set of titration experiments were carried out, which was summarized in Figure 3. When monitored at 395nm, Ni^{2+} gave good absorbance-concentration linearity [Fig. 3A]. The peak wavelength shifted gradually from 395nm to 384nm with the titration with EDTA, and from 384nm to 390nm (not 395nm) with the titration with Ni^{2+}. It was found that excess amount of EDTA does not interfere with the absorbance reading of Ni-EDTA, which was peaked at 1:1 (EDTA/Ni^{2+}) molar ratio and then reduced to a stable reading when the molar ratio is higher than 2:1 (EDTA/Ni^{2+}) [Fig. 3B]. It is not known yet why the absorbance is higher when the molar ratio of EDTA/Ni^{2+} is between 1:1 and 2:1. On the other hand, excess amount of Ni^{2+} led to increase in absorbance reading. The intercept of such increase is much lower when molar ratio of Ni^{2+}/EDTA is greater than 1:1, which is due to weaker absorbance of excess Ni^{2+}, compared to Ni-EDTA [Fig. 3C].

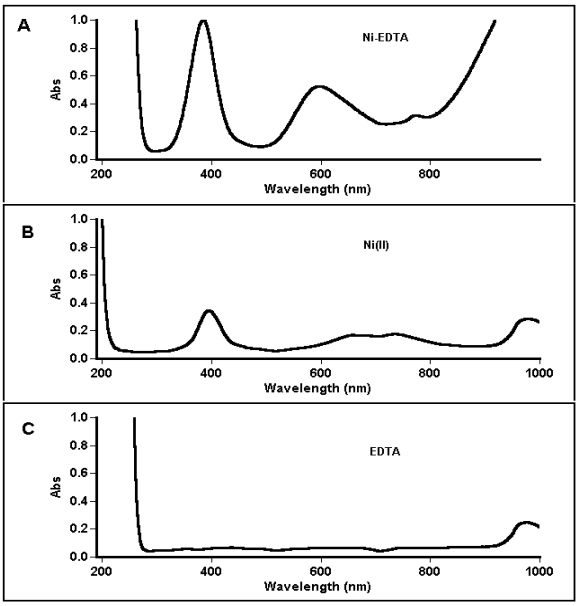

Figure 2. The UV-visible absorbance spectra of 50mM Ni-EDTA (A), $NiSO_4$ (B) and EDTA (C).

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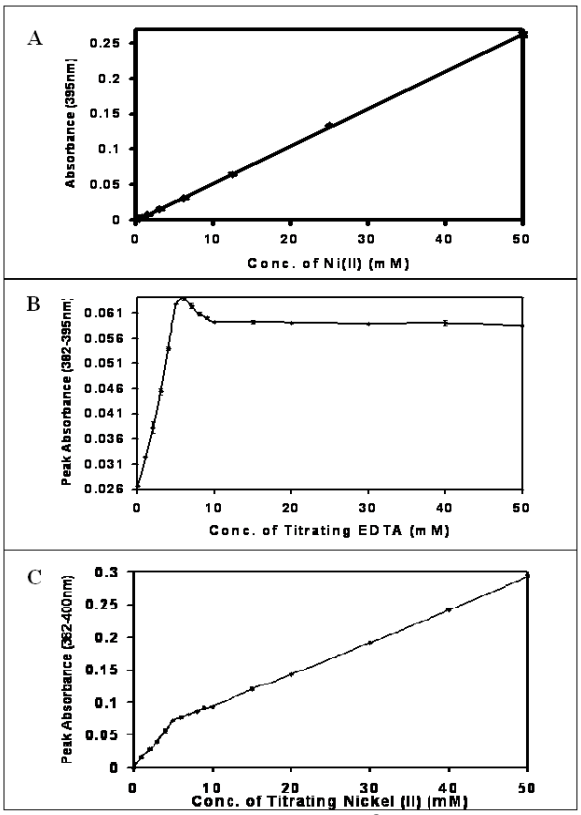

Figure 3. The peak absorbance of Ni^{2+} at concentrations from 0 to 50mM (A); 5mM of Ni^{2+}, titrated with 0 – 50mM EDTA (B); and 5mM of EDTA, titrated with 0 – 50mM Ni^{2+} (C). Average of duplicate measurements was plotted with the error bar shown. Linear trending line was used to line up the dots in A.

The wavelength scanning and titration made it possible to develop a quick nickel assay to track the loss of Ni^{2+} during IMAC, as described in Materials and Methods section. The results were summarized in Table 1. There was no significant loss of Ni^{2+} during the chromatography with PBS buffer or *E. coli* whole cell extract under the conditions used. However, the EDTA/Mg^{2+} treatment resulted in loss of about half of the Ni^{2+} from the column. In addition to the microplate assay, the chromatogram of EDTA stripping could be used to monitor the A_{384} peak of Ni-EDTA coming off the column [Fig. 4]. By comparing the Ni-EDTA peak area before and after IMAC, the loss of Ni^{2+} during IMAC could be estimated. Recently, we observed that imidazole forms a blue complex with Ni^{2+}, which could interfere with the Ni-EDTA assay. The interference could be minimized by: 1) washing extensively to remove excess imidazole left in the column; 2) add an imdazole "elution" step after charging the fresh column to mimic the IMAC elution condition.

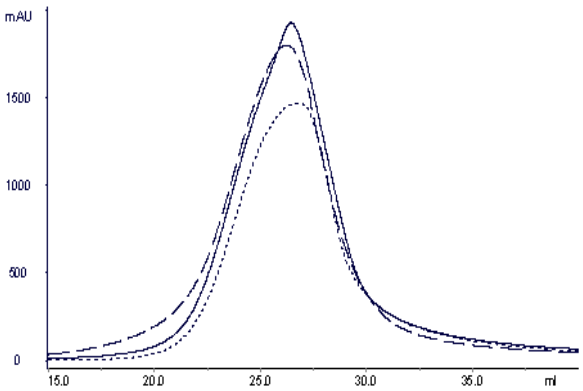

Figure 4. Chromatogram at 384nm of EDTA stripping of a Ni-chelating Sepharose Fast Flow column (column diameter: 1.6cm, bed height: 10cm) before and after IMAC purification of a polyhistidine-tagged protein from *E. coli* whole cell extract. Before IMAC, the Ni-column was stripped either directly with 100mM EDTA (light dashed line, peak area: 9837mAU.ml) or with 100mM EDTA after an imidazole "elution" step mimicking the IMAC elution condition (solid line, peak area: 12137mAU.ml). After IMAC, the column was stripped directly with 100mM EDTA (heavy dashed line, peak area: 12067mAU.ml). Apparently, the loss of Ni(II) in IMAC is less than 1%.

Table 1. Leaching of Ni(II) in IMAC measured by microplate Ni(II) assay (average of 3 measurements, CV < 10%).

Experimental conditions	Ni2+ capacity in 5ml Ni Sepharose HP column (µmole)		% Ni^{2+} leached
	Before IMAC	After IMAC	
Direct EDTA stripping	122.0	n/a	n/a
IMAC with PBS buffer	127.3	123.8	2.7
IMAC with PBS buffer, treated with EDTA/Mg^{2+}	128.4	64.3	49.9
IMAC with E. coli whole cell extract	122.9	121.8	0.0

SUMMARY

In summary, by complexing Ni^{2+} with EDTA, a spectrophotometric based assay was developed for routine measurement of Ni^{2+} loss during IMAC. The assay could be carried out in either microplate format or chromatography format. Though this assay could meet our need for rapid estimation on Ni^{2+}

concentration, it is not intended as an alternative to other nickel determination techniques, such as atomic absorption spectrometry (AAS).

ADDENDUM

This work was completed in 2001 during the development of IMAC purification using EDTA/Mg^{2+} to increase the binding selectivity for recombinant proteins [3]. Two recent papers described monitoring Ni^{2+} leakage during IMAC using UV-vis determination. Hu et al. [5] determined the Ni^{2+} concentration in EDTA solution at 400nm. From the UV-visible spectrum obtained (Figure 1, Panel A), it is clear that the peak absorbance shifted from 395nm of free Ni^{2+} to 384nm of Ni-EDTA., thus absorbance should be measured at 384nm, rather than 400nm. Chen et al. [6] measured the leached Ni^{2+} concentration using UV-vis at 800nm. Our results showed that there is no absorbance peak at 800nm, therefore, this is not a good method for determine the Ni^{2+} concentration.

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